Relative virulence of *Staphylococcus aureus* bovine mastitis strains representing the main Canadian *spa* types and clonal complexes as determined using in vitro and in vivo mastitis models

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ABSTRACT

Staphylococcus aureus is one of the main pathogens leading to both clinical and subclinical bovine mastitis in dairy cattle. Prediction of disease evolution based on the characteristics of *Staph. aureus* isolates that cause intramammary infections and understanding the host-pathogen interactions may improve management of mastitis in dairy herds. For this study, several strains were selected from each of the 6 major Canadian *spa* types associated with mastitis (t267, t359, t529, t605, t2445, and t13401). Adherence to host cells and intracellular persistence of these strains were studied using a bovine mammary gland epithelial cell line (MAC-T). Additionally, relative virulence and host response (cytokines production) were also studied in vivo using a mouse model of mastitis. Whole-genome sequencing was performed on all strains and associations between clonal complex, sequence type, and presence of certain virulence factors were also investigated. Results show that *spa* type t2445 was correlated with persistence in MAC-T cells. Strains from *spa* t359 and t529 showed better ability to colonize mouse mammary glands. The exception was strain sa3154 (*spa* t529), which showed less colonization of glands compared with other t359 and t529 strains but possessed the highest number of superantigen genes including *tst*. All strains possessed hemolysins, but *spa* types t529 and t2445 showed the largest diameter of β -hemolysis on blood agar plates. Although several *spa* types possessed 2 or 3 serine-

aspartate rich proteins (Sdr) believed to be involved in many pathogenic processes, most t529 strains expressed only an allelic variant of *sdrE*. The *spa* types t605 (positive for the biofilm associated protein gene; *bap+*) and t13401 (*bap-*), that produced the largest amounts of biofilm in vitro, were the least virulent in vivo. Finally, strains from *spa* type t529 (ST151) elicited a cytokine expression profile (TNF- α , IL-1 β and IL-12) that suggests a potential for severe inflammation. This study suggests that determination of the *spa* type may help predict the severity of the disease and the ability of the immune system to eliminate intramammary infections caused by *Staph. aureus*.

Key words: *Staphylococcus aureus*, *spa* type, bovine mastitis, intramammary infection, host immune response

INTRODUCTION

Bovine mastitis is an inflammation of the mammary gland most often caused by a bacterial IMI. The disease may be asymptomatic or symptomatic (subclinical or clinical mastitis), persistent (chronic) or nonpersistent. Bovine mastitis places a considerable economic burden on the world dairy industry. The cost is estimated at 660 CAD (US\$520 at time of writing) per lactating cow per year for Canadian dairy farms (Aghamohammadi et al., 2018). In Canada, *Staphylococcus aureus* is the most prevalent pathogen recovered from clinical mastitis and the second most frequently isolated bacteria, after non-*aureus* staphylococci, from apparently healthy cows (Reyher et al., 2011). Chronic *Staph. aureus* IMI may last several weeks during lactation (Pichette-Jollette et al., 2019), as pathogen clearance by the host immune system is difficult (Côté-Gravel and Malouin, 2019) and antibiotic treatment is minimally effective (Ster et al.,

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2017) due to virulence factors promoting cell invasion (Proctor et al., 2006) and biofilm formation (Melchior et al., 2007; Jacques et al., 2010).

Biofilm production by *Staph. aureus* also influences manifestations of the disease. Biofilms are extracellular matrices that minimize antibodies opsonization and phagocytosis. Bacteria within biofilms are also less susceptible to antibiotics (Jacques et al., 2010). *Staphylococcus aureus* can produce biofilms of various kinds. The polysaccharide intercellular adhesin found in biofilms is primarily composed of exopolysaccharides [poly- β (1–6)-*N*-acetylglucosamine] synthesized by the *icaABDC* operon, whereas the *bap* gene can form a protein-based biofilm (biofilm associated protein; Snel et al., 2015; Taglialegna et al., 2016). The quorum-sensing system Agr is one of the most significant regulatory mechanisms controlling biofilm formation in *Staph. aureus* (Arciola et al., 2015). Low activation of the Agr system results in high biofilm production (Otto, 2013).

During the establishment of the infection, *Staph. aureus* can utilize one or more of its microbial surface components recognizing adhesive matrix molecules (MSCRAMM) to interact with and colonize mammalian hosts. Serine-aspartate rich (Sdr) proteins are virulence factors that are part of the MSCRAMM family in *Staph. aureus* (Foster et al., 2014; Foster, 2019), and their implication in biofilm formation, adhesion to host molecules and immune evasion mechanisms have been previously described (Barbu et al., 2014; Sharp et al., 2012). Capsular polysaccharides (CPS) produced at the surface of bacteria and the CPS types may influence virulence and host defenses (O’Riordan and Lee, 2004). The CPS types (mainly CP5 or CP8) or the level of capsule expression are among the proposed characteristics enabling *Staph. aureus* to cause lasting infections.

During colonization of the mammary gland, *Staph. aureus* can produce several virulence factors to sustain infection and promote its survival in the host. These virulence factors are generally associated with damage to the mammary gland epithelium and local inflammation and are also associated with clinical signs (Rainard et al., 2018). In addition, enterotoxins, enterotoxins-like and TSST-1 (coded by the *tst* gene) are superantigens (SAg) which, when expressed by the bacterium, may stimulate one or more T lymphocyte subfamilies. This results in an uncontrolled release of pro-inflammatory mediators that greatly affects efficacy of the immune response (Wilson et al., 2018).

Cytokines and chemokines are soluble proteins or glycoproteins secreted by a wide variety of cells such as immune (e.g., neutrophils) and epithelial cells (Oviedo-Boyso et al., 2007). The Holstein breed, which

is more susceptible to infections, is more likely to develop chronic inflammation due to a high production of pro-inflammatory cytokines such as IL-1 β and tumor necrosis factor (TNF)- α compared with other breeds such as the Rendena breed (Curone et al., 2018). These 2 cytokines are often associated with IMI caused by *Staph. aureus* (Oviedo-Boyso et al., 2007; Bannerman et al., 2004, 2008; Bannerman, 2009). Two other cytokines that have been associated with IMIs caused by *Staph. aureus* are pro-inflammatory IL-12 that induces IFN- γ (Mühl and Pfeilschifter, 2003) involved in the activation of neutrophils and macrophages (Bannerman et al., 2004, 2008; Bannerman, 2009), and that promotes the Th1 type cell-mediated immune response (Trinchieri, 1997; Bannerman et al., 2008). Concentrations of certain growth factors normally present in a healthy gland, such as transforming growth factor (TGF)- α and TGF- β 1, which are respectively pro- and anti-inflammatory, are increased in milk during an IMI caused by *Staph. aureus* (Bannerman et al., 2008).

Studies on the correlation of virulence factors of bovine isolates to various disease manifestations have met with incomplete success (Veh et al., 2015; Pichette-Jollette et al., 2019; Hoekstra et al., 2020; Naushad et al., 2020). There are many reasons for this partial correlation. On the one hand, not all *Staph. aureus* strains possess all virulence factors and if several are present, they may act synergistically. On the other hand, because it is difficult to control the homogeneity of the cow population, and because the immune response of each cow in observational studies varies greatly according to parity, DIM, and genetic background (Bannerman et al., 2008; Canning et al., 2017; Ruiz et al., 2017; Curone et al., 2018), any association between bacterial virulence and clinical outcome in cows is very difficult to establish. It would be prohibitively expensive to conduct an experiment in which *Staph. aureus* lineages with or without particular virulence factors were randomly assigned to a standardized cow population to assess clinical outcomes. This is why the present study aimed to compare virulence-associated phenotypes of *Staph. aureus* strains most commonly recovered from quarter milk samples using assays and conditions that could be monitored and controlled in the laboratory. The phenotypes studied included adhesion to and persistence within bovine mammary gland epithelial cells in vitro, whereas the ability to colonize the mammary gland and the host cytokine expression response to *Staph. aureus* colonization were studied in a mouse mastitis model. Also, because *Staph. aureus* strains are grouped into specific clonal lineages each possessing specific sets of virulence factors and remarkably similar genomes because of a highly developed restriction-modification system that

prevents horizontal gene transfer among lineages (Park et al., 2020), we used *spa* typing to represent the different types of strains to be compared in this study. The *spa* gene is indeed one of the most useful single genetic markers for distinguishing *Staph. aureus* lineages (Aires-de-Sousa et al., 2006; Rodriguez et al., 2015). Results show that determining the *spa* type of mastitis isolates may help predict disease outcome in cows.

MATERIALS AND METHODS

Bacterial Strains

Twenty-three *Staph. aureus* strains from quarter milk samples partly characterized by Pichette-Jollette et al. (2019) were used in this study. The strains were obtained from the Mastitis Pathogen Culture Collection of the Canadian Bovine Mastitis and Milk Quality Research Network (Faculté de Médecine Vétérinaire, Université de Montréal, St-Hyacinthe, QC, Canada). These strains were collected from 91 Canadian herds, participating in the National Cohort of Dairy Farms located in the Atlantic Provinces, Quebec, Ontario, and Western Canada, and followed for 2 years (2007 and 2008). All *Staph. aureus* strains were from quarter milk samples with at least one colony detected in a 10- μ L sample (100 cfu/mL). The *Staph. aureus* species were confirmed by a positive catalase test, hemolysis on blood agar, and positive nuclease and coagulase tests. All strains collected were archived and a barcode was assigned in the database with all related information at the farm, cow, and quarter level (Reyher et al., 2011).

The 23 strains were chosen to represent the most prevalent *spa* types found in Canadian dairy cattle (Veh et al., 2015; Pichette-Jollette et al., 2019). The 6 most prevalent *spa* types were t529 ($n = 115$), t267 ($n = 67$), t359 ($n = 36$), t605 ($n = 18$), t2445 ($n = 11$), and t13401 ($n = 10$) (Pichette-Jollette et al., 2019). To avoid picking nonrepresentative outliers, we used the already available data on biofilm production as guidance for strain selection (Pichette-Jollette et al., 2019). We made sure the in vitro biofilm production of selected strains was within the interquartile range of each *spa* type population. Hence, within this interquartile range, 3 strains were randomly picked for *spa* types t267, t359, t605, and t2545. On the other hand, for *spa* type t529, which was overrepresented in Canadian bovine strains (collection of $n = 115$), we randomly picked 6 *tst*-negative and 1 *tst*-positive strains (Table 1). Although only 8% of the 115 t529 isolates were positive for the *tst* gene, we wanted to make sure that at least one strain possessing such a superantigen gene was included in the study. Noteworthy, *spa* types t605

and t2445 were described as strong and weak biofilm producers, respectively (Pichette-Jollette et al., 2019). Strains from *spa* type t13401 were also strong biofilm producers but because they differed phenotypically and genotypically from the other 9 strains of the clonal complex (CC) CC97 based on biofilm production and on the presence/absence of the *lukM* gene, respectively, (Table 1), 4 strains (instead of 3) were chosen and studied for that group. Clonal complex CC97 is among the most widespread CC in Europe (Hoekstra et al., 2020), and strains producing the LukMF' leukocidin (encoded by *lukMF*) have been associated with bovine lineages of *Staph. aureus* (Vrieling et al., 2016; Hoekstra et al., 2018).

Whole-Genome Sequencing, Assembly, and Annotation

Staphylococcus aureus strains were inoculated in 3 mL of brain heart infusion medium (Becton Dickinson). The GenElute Bacterial Genomic DNA Kit (Sigma-Aldrich) was used to extract bacterial DNA as described before (Pichette-Jollette et al., 2019). Illumina sequencing libraries were then prepared from 100 ng of DNA using the QIAGEN QIAseq FX DNA Library Kit for Illumina instrument according to the manufacturer (QIAGEN). All purifications were made on double solid phase reversible immobilization using carboxyl-coated magnetic beads with an adjusted ratio. Desired fragment sizes were between 600 bp and 800 bp and obtained by treatment with the double-stranded DNA Shearase enzyme (Zymo Research) according to the manufacturer. Libraries were prepared according to Baby et al. (2018) and validated using a 2100 Bioanalyser (Agilent Technologies). A PicoGreen fluorometric assay was used to quantify gDNA before pooling libraries. Sequencing was performed on an Illumina HiSeq 2000 sequencing system at the Plateau de Biologie Moléculaire et Génomique Fonctionnelle of the Institut de Recherche Cliniques de Montréal (Montreal, QC, Canada). Samples were multiplexed in a single sequencing lane, and approximately 1 million to 2 million paired-end reads of 50 bp were obtained for each library.

The resulting reads were de novo assembled using a custom assembly pipeline based on the Roche Newbler Assembler (version 2.9, <http://sequencing.roche.com>). Briefly, sequencing reads were trimmed for adaptor sequences and base call quality established using Trimmomatic v0.36 LEADING:20 TRAILING:20 SLIDINGWINDOW:4:20 MINLEN:36 (Bolger et al., 2014). Trimmed reads were then de novo assembled and in parallel assembled using staph_aureus_RF122

Table 1. Characteristics of *Staphylococcus aureus* strains used in this study and representing the major *spa* types found in Canadian dairy herds

<i>spa</i> type ¹	ID ²	Barcode ³	GenBank ⁴	CC ⁵	ST ⁵	<i>lukM</i> ⁶	<i>seq</i> and others ⁷	<i>tst</i> and others ⁸	<i>bap</i> ⁹	CPS type ¹⁰	Agr group ¹¹	Biofilm ¹²
t267	sa3003	20902889	SRR11471960	CC97	ST352	+	—	—	—	5	I	0.9
	sa3353	32400168	SRR11471959	CC97	ST352	+	—	—	—	5	I	0.8
	sa3493	40913568	SRR11471962	CC97	ST352	+	—	—	—	5	I	1.1
	sa3014	31312165	SRR11471958	CC97	ST352	+	—	—	—	5	I	0.7
t359	sa3022	31310741	SRR11471957	CC97	ST352	+	—	—	—	5	I	1.5
	sa3199	20213169	SRR11471956	CC97	ST352	+	—	—	—	5	I	2.9
	sa2539	31301213	SRR11471965	CC151	ST151	+	+	—	—	8	II	1.0
	sa2978	30210004	SRR11471964	CC151	ST151	+	+	—	—	8	II	1.1
t529	sa3154	11200086	SRR11471968	CC151	ST351	+	+	+	—	8	II	0.9
	sa3213	32002607	SRR11471970	CC151	ST151	+	+	—	—	8	II	0.8
	sa3370	32410242	SRR11471963	CC151	ST151	+	+	—	—	8	II	1.1
	sa3498	40705125	SRR11471966	CC151	ST151	+	+	—	—	8	II	1.0
t605	sa3511	41610503	SRR11471969	CC151	ST151	+	+	—	—	8	II	1.1
	sa3129	10214695	SRR11471990	CC126	ST2270	—	—	—	+	5	II	1.9
	sa3131	10214701	SRR11471991	CC126	ST2270	—	—	—	+	5	II	3.2
	sa3137	10213728	SRR11471992	CC126	ST2270	—	—	—	+	5	II	2.9
t2445	sa3454	40312927	SRR11471984	CC97	ST352	+	—	—	—	5	I	0.7
	sa3456	40300122	SRR11471983	CC97	ST352	+	—	—	—	5	I	0.8
	sa3468	40812250	SRR11471982	CC97	ST352	+	—	—	—	5	I	1.2
	sa2117	10705001	SRR11471989	CC97	ST2187	—	—	—	—	5	I	3.2
t13401	sa2911	21808432	SRR11471986	CC97	ST2187	—	—	—	—	5	I	4.3
	sa2925	32702828	SRR11471988	CC97	ST2187	—	—	—	—	5	I	3.6
	sa3071	21110375	SRR11471985	CC97	ST2187	—	—	—	—	5	I	3.3

¹The *spa* type data are from Pichette-Jollette et al. (2019).

²ID is the strain identification number used in this study.

³The barcode refers to the NCDF databank annotation (see Materials and Methods).

⁴Genome sequences are available through the listed Genbank numbers (<https://www.ncbi.nlm.nih.gov/genbank/>).

⁵CC (clonal complex) and ST (sequence type) data are from Park et al. (2020).

⁶The *lukM* data are from Pichette-Jollette et al. (2019).

⁷The *seq* and other enterotoxin genes (*sec*, *sei*, *selk*, *selm*, *seln*, *selo*) data are from Park et al. (2020), and Pichette-Jollette et al. (2019).

⁸The *tst*_{hprt} and other enterotoxin genes (*sec*_{hprt}, *selk*_{hprt}, *selm*_{hprt}) data are from Park et al. (2020), and Pichette-Jollette et al. (2019).

⁹*bap*, coding for the biofilm associated protein.

¹⁰CPS (capsular polysaccharide) type data are from Park et al. (2020).

¹¹Agr (autoinducing peptide) group data are from Park et al. (2020).

¹²Biofilm data from Pichette-Jollette et al. (2019). The value corresponds to the relative amount of biofilm measured by crystal violet staining. Biofilm production was normalized to that observed for strain sa2539 (t529), which produced a stable and moderate amount of biofilm.

(NC_007622.1) as the reference. Both assembly predictions were then combined using a custom method in which all reads and predicted de novo and reference based contigs were reassembled using the Roche Newbler Assembler (version 2.9). Assembled contigs were annotated using GeneMarks version 4.28 (Besemer et al., 2001).

Blast (version ncbi-blast 2.7.1+; <https://blast.ncbi.nlm.nih.gov/Blast.cgi>) was done against the UniProt *Staph. aureus* proteome (<https://www.uniprot.org/uniprot/?query=taxonomy:1280>) to identify unique genes and family genes. Core genome was filtered with >80% coverage to highlight unique genes for each *spa* type and each strain. Filtering was done according to the protein sequence of genes (around 3,650 proteins annotated). The multilocus sequence typing was done using Simulate PCR (Gardner and Slezak, 2014) to obtain sequence of the 7 *Staph. aureus* house-keeping gene (*arcC*, *aroE*, *glpF*, *gmk*, *pta*, *tpi*, and *yqi*). Their sequences were submitted on PubMLST (<https://pubmlst.org/saureus/>) to obtain allelic profiles of genes and to determine the sequence type (ST) of *Staph. aureus* strains.

To examine strain similarities, a phylogenetic tree and heatmap representation of gene compositions for the 23 *Staph. aureus* genomes were created. Genes were predicted by Roary (version 3.13.0) using Prokka version 1.14.0 (<https://sanger-pathogens.github.io/Roary/>). A total of 3,759 genes were annotated using this pipeline. A total of 2,385 core genes were identified by Roary. A heatmap was generated, using R v3.6.3 (<https://r-project.org>) and the gplots package, using the absence or presence matrix of genes produced by Roary. *Staph. aureus* strains were clustered (complete method) by gene presence and absence using Manhattan distance.

Hemolysis Assay

Hemolysis by *Staph. aureus* strains was visualized as previously described (Herbert et al., 2010; Mitchell et al., 2013). Hemolysis was measured because production of the α -toxin (Hla) can affect the duration of intracellular persistence by causing cell lysis, which can lead to tissue damage and inflammation (Soe et al., 2021), affecting the phenotypes observed in subsequent assays. Briefly, bacterial suspensions were adjusted to a 0.5 McFarland turbidity standard and were spotted on sheep blood agar plate (Oxoid Microbiology Products). Plates were incubated for 24h at 35°C. After incubation, plates were transferred to 4°C for an additional 24 h. Zone diameters (3 replicates per strain) of β -hemolysis were measured.

Staph. aureus Cellular Adherence and Intracellular Persistence

A bovine mammary epithelial cell line (MAC-T cells) was used to measure cellular invasion (Moisan et al., 2006). The MAC-T cells were maintained in Dulbecco's modified Eagle medium (Wisent) supplemented with 10% heat-inactivated fetal bovine serum, 1× sodium pyruvate (Wisent), 1× antibiotic and antifungal solution (Wisent), 5 μ g/mL insulin (Roche Diagnostics Inc.) and 1 μ g/mL hydrocortisone (Sigma). Cells were incubated at 37°C with 5% CO₂.

The assay was performed as previously described (Côté-Gravel et al., 2016) with minor modifications. Briefly, 48 h before inoculation, $\sim 2 \times 10^5$ cells/mL were added in treated Cell-BIND 24-wells plate (Corning) to obtain a minimum of 50% confluence the next day. The growth medium was replaced by the invasion medium (IM), a growth medium without antibiotics containing 1% heat-inactivated fetal bovine serum, 20 to 24 h prior inoculation. Twenty-four well plates were incubated at 37°C with 5% CO₂ until 100% confluence (monolayer cells). Before cell infection, an overnight culture of the test bacterium was diluted 1:20 in fresh tryptic soy broth medium and grown under agitation to mid-logarithmic growth phase, then washed twice with PBS and diluted in IM to obtain a multiplicity of infection of 50 corresponding to $\sim 2 \times 10^7$ bacteria. Infected monolayers were incubated at 37°C with 5% CO₂. Bacterial adherence to cells was measured after 30 min of incubation. Cells were then washed 3 times with Dulbecco's PBS (D-PBS) to remove unbound bacteria and were suspended by using trypsin and lysed with 0.05% Triton X-100. Bacterial cfu was determined by serial dilution on trypticase soy agar (TSA) plates.

Intracellular persistence of bacterial strains was evaluated 20 h postinfection. After 3 h of invasion time, monolayers were washed with Dulbecco's modified Eagle medium and incubated 17 h in IM supplemented with 20 μ g/mL lysostaphin (Moisan et al., 2006; Tuscherr et al., 2010) and 50 μ g/mL gentamicin to kill extracellular bacteria. After 20 h of infection, cells were washed 3 times with D-PBS, and cells were detached from the plate using trypsin and lysed with 0.05% Triton X-100. Bacterial cfu was determined by serial dilution on TSA plates.

A correction was applied on the measured Log₁₀ cfu/wells to account for MAC-T cell mortality. The MAC-T cell viability was assessed by the reduction of 3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide (MTT) into insoluble formazan crystals by mitochondria (as described below). Results were expressed using the following formula:

$$\frac{\text{Log}_{10} \text{ bacterial cfu/wells /}}{(\text{Absorbance of MAC-T cells [MTT assay]})}$$

– Absorbance of bacterial background [MTT assay]),

where the bacterial background was the absorbance (MTT assay) of infected cells that were treated with 0.05% Triton X-100 for 10 min before MTT addition (i.e., the absorbance only due to bacteria).

Staph. aureus Cytotoxicity Assay

The cytotoxicity of *Staph. aureus* strains for MAC-T was measured by the MTT assay. The assay was performed as described by Kubica et al. (2008) and Côté-Gravel et al. (2016) with minor modifications. Briefly, *Staph. aureus* infection of MAC-T cells was achieved as described above for the cell invasion assay, without inducing lysis of cells after 30 min or 20 h of infection. After each infection time (30 min and 20 h), cells were washed as described before and then incubated 2 h with 100 μL of MTT reagent (5 mg/mL; Sigma) in D-PBS at 37°C. Then, cells were lysed, and formazan crystals were solubilized overnight by using an acidic solvent solution of 16% SDS and 40% DMF, pH 4.7. Each well was read using an Epoch microplate reader (Biotek Instrument Inc.) at a wavelength of 570 nm. All measurements were performed in duplicate, and 2 controls were included in each plate: a high cell viability control (cells cultured without bacteria) and a lysed, infected cells control (bacterial background control) where infected cells were treated with 0.05% Triton X-100 for 10 min before MTT addition. The level of metabolic activity was calculated using the following formula:

$$\frac{[(\text{Absorbance of infected cells}) - (\text{Absorbance of bacterial background})]}{\text{High cell viability}} \times 100.$$

Mouse Intramammary Infection Model

The animal experiments were conducted following the guidelines of the Canadian Council on Animal Care and the institutional ethics committee on animal experimentation of Université de Sherbrooke. The institutional ethics committee on animal experimentation of the Faculté des Sciences of Université de Sherbrooke specifically approved the murine mastitis model and the present study protocol (2017–1966 FM2017–01B).

Intramammary infections were performed as described in Brouillette et al. (2004). Briefly, the 12- to

14-d offspring were removed 1 h before the infection of lactating CD-1 mice (Charles River Laboratories, QC, Canada). Mice were anesthetized with ketamine and xylazine at 83 and 13 mg/kg of BW, respectively, and 2 mammary glands, right (R4) and left (L4), were inoculated under a binocular. Each mammary duct was exposed by a small cut before injection of 100 μL of the tested bacterial suspension (10^3 cfu/mL) in endotoxin-free D-PBS. Five to 6 glands were infected per test strain. Mammary glands were removed aseptically at 10 h or 20 h after infection, depending on the experiment (specified in the figure legends). Glands were easily detached from the skin using small forceps and were completely removed without any contamination from surrounding tissues. Each gland, which holds in one piece, was weighed and inspected for inflammation and redness. Glands were then homogenized in 50 mM potassium phosphate buffer pH 6. A fraction was kept aside for the myeloperoxidase (MPO) assay (see below). A 10 $\times$ saline solution was diluted 1:10 in the remaining gland homogenate to determine the bacterial load and for detection of cytokines and chemokines. The bacterial load (cfu) was evaluated by serial dilution in PBS and plating on TSA. The cytokine and chemokine assays are described below.

MPO Assay

Neutrophil recruitment in mammary tissues was measured by quantification of the MPO enzyme activity, which served as an indicator of inflammation, as previously described (Kubica et al., 2008; Côté-Gravel et al., 2016). Briefly, 4 $\times$ cetrirmonium bromide buffer was added to a 500- μL fraction of mammary gland homogenate at a dilution of 1:4. Samples were vortexed for 30 s and sonicated for 2 min in a sonication bath. Then, a freeze-thaw step in liquid azote was applied on samples. Samples were centrifuged at 4°C for 15 min in 2 000 $\times g$. Those supernatants were further centrifuged at 4°C for 15 min at 15,000 $\times g$ and aliquoted and stored at –80°C before performing the MPO assays.

In 96-well plates, 10 μL of each mammary gland extract in 50 mM cetrirmonium bromide (1 $\times$) phosphate buffer 50 mM, pH 6, were incubated with a solution of *o*-dianisidine hydrochloride (167 $\mu\text{g/mL}$) and 0.0005% H_2O_2 (Sigma). An Epoch microplate reader (Biotek Instruments Inc.) was used to kinetically measure MPO activity at 460 nm with intervals of 15 s over a period of 5 min. All results were expressed as units of MPO per gland. A unit of MPO corresponding to the amount of enzyme that degrades 1 μmol of H_2O_2 /min at 25°C, using the absorption coefficient of 11.3 $\text{mM}^{-1}\text{cm}^{-1}$ for

o-dianisidine. Results were expressed as units of MPO per gram of gland.

Preparation of Mammary Gland Homogenates for Cytokines and Chemokines Assays

A fraction of mammary gland homogenates (5–6 infected glands per strain) was prepared for dosage of cytokines and chemokines using the mouse cytokine-chemokine array 31-Plex (Eve Technologies, Calgary, AB, Canada). Samples were prepared by adding a 1:100 dilution of protease inhibitors to mammary gland homogenates. Samples were kept on ice and then centrifuged twice 15 min at $10,000 \times g$ at 4°C. Supernatants were stored at –80°C until used. The amount of proteins was measured for each sample by using the BCA kit (Pierce, Thermo-Fisher). Sample concentrations were harmonized to the lowest protein concentration measured and were then analyzed by Eve Technologies. Before analysis, corrections were applied on all data set to account for dilutions and weight of mouse mammary glands. Median of cytokine concentrations for each *spa* types were calculated. Concentration values were expressed in picograms per gram of mouse mammary gland.

Statistical Analyses

Statistical analyses were carried out with the GraphPad Prism software version 8 (GraphPad Software Inc.). Bacterial counts (cfu) were transformed in base 10 logarithm values before statistical analysis. Statistical tests used for the analysis of each experiment and significance are specified in the figure legends. The different levels of cytokines and chemokines induced by mouse IMI were categorized by using a principal component analysis (PCA) using the XLSTAT software for Excel (XLSTAT 2019, Microsoft Corp.). The principal components displaying the highest and second-highest percentage of variables were used. Both axes were chosen depending on the percentage of variability displayed by the scree plot. Pearson correlation was used to calculate the relationship between the variables and significance was fixed at 5%.

RESULTS

Strains Relatedness

The general characteristics of *Staph. aureus* strains used in this study are shown in Table 1. A global analysis of whole-genome sequences is reported elsewhere (Park et al., 2020), and Table 1 summarizes the rel-

evant information for the present study such as the CC and ST. The phylogenetic tree for the observed clades is shown in Figure 1, along with the relative similarity of the studied strains within each clade. Strains from *spa* types t267 and t359 (all CC97 and ST352) were found to be closely related. Other strains from CC97 and ST352 strains, such as t2445, were related as well, whereas those from *spa* types t13401 (CC97 and ST2187), were more distantly related to the other CC97 strains. The strain sa2117 of *spa* type t13401 was the exception, as it differed significantly from the rest of its *spa* group. All CC97 strains possessed the *lukM* gene, except for t13401 strains, and were of capsular type 5 and of the Agr group I. Strains from *spa* types t605 and t529 (all of Agr group II) were even more distant and were from clades CC126 (ST2270) and CC151 (ST151), respectively. The *spa* type t529 (capsular type 8) was the most commonly found among bovine mastitis isolates in Canada (Pichette-Jollette et al., 2019), and expresses several SAg genes, including *seg*, *sec*, *selk*, *selm*, *seln*, and *selo* (Table 1). In this group, strain sa3154 (CC151, ST351) was quite distinct (Figure 1), and contains additional enterotoxin/SAg genes (*tst_{bov}*, *sec_{bov}*, *seil_{bov}*) associated with *Staph. aureus* pathogenesis island SaPI_{bov}. As t13401, the *spa* type t605 was negative for *lukM*. The biofilm associated protein (Q79LN3_STAAU) gene (*bap*) was present only in t605 strains (capsular type 5). Strains from *spa* types t605 and t13401 produced the highest levels of biofilm although t13401 strains did not have the *bap* gene. Strains from t2445 produced the lowest level of biofilm (Table 1).

Given the redundancy of Sdr proteins in *Staph. aureus* (SdrC [WP_001060440.1], SdrD [WP_000934474.1], SdrE [WP_000610294.1]) and hence their apparent importance in virulence (Sharp et al., 2012; Barbu et al., 2014), the distribution and sequence homologies of 3 Sdr genes (*SdrC*, *SdrD*, and *SdrE*) were specifically investigated among the studied strains (Table 2). Gene *sdrC* was found in all strains except one (strain sa3042) from the t13401 group (Table 2). All strains from t605 and t529 had mutations leading to an early stop codon in *sdrC* (Supplemental Figure S1, <https://doi.org/10.6084/m9.figshare.14981319.v1>). Also, there was a deletion of 330 bases in the *sdrC* gene of t2445 strains occurring in a repeated region of the protein rather than in the functional region (Supplemental Figure S1). The *sdrE* gene was found in all *spa* types except for t13401. However, 2 strains of *spa* type t529 had no *sdrE* gene and an allelic variant of *sdrE* was noticed in the 5 other studied strains of that group (Table 2 and Supplemental Figure S1). Very similar sequences of SdrD were found in 3 *spa* types (t267, t2445 and t359).

Figure 1. Phylogenetic tree and heatmap representation of gene compositions for the 23 *Staphylococcus aureus* genomes analyzed in this study. Genes were predicted using Roary and a total of 2,385 genes were defined as core genes. The heatmap was generated using R v3.6.3 and the gplots package using the absence or presence matrix of genes produced by Roary. Genes are represented by white and black bar for absence and presence, respectively. *Staphylococcus aureus* strains were clustered (complete method) by gene presence and absence using Manhattan distance; clustering dendrograms are shown on the left. On the right, the *spa* type is shown using a black box and clonal complexes (CC) and sequence types (ST) are indicated following the strain numbers.

Table 2. Distribution of *sdr* genes among most prevalent *Staphylococcus aureus spa* types

<i>spa</i> Type	<i>sdrC</i>	<i>sdrD</i>	<i>sdrE</i>
t267	5/5	5/5	5/5
t359	3/3	3/3	3/3
t529 ¹	7/7	0/7	5/7
t605 ²	5/5	0/5	4/5
t2445 ³	3/3	3/3	3/3
t13401	12/13	0/13	0/13

¹All strains presented an early stop codon in *sdrC*. All 5 strains of *sdrE* were allelic variants, and 2 strains (sa3511 and sa3370) had no *sdrE* gene detected in their genomes.

²All strains presented an early stop codon in *sdrC*.

³All strains presented a deletion of 330 bases in the repeated region of *sdrC* (see Supplemental Figure S1, <https://doi.org/10.6084/m9.figshare.14981319.v1>).

Staph. aureus Hemolytic Activity, Cellular Adherence, and Intracellular Persistence

Since production of hemolysins plays a role in bacterial cytotoxicity in cell culture and will also influence the inflammation process in the mammalian host (Soe et al., 2021), *Staph. aureus* strains were first compared for their hemolytic activity on blood agar. All studied strains were capable of producing a β -hemolysis, however, the amount of hemolysin varied depending on the clonal origin of the strains (Figure 2). The *spa* types t529 and t2445 displayed the largest hemolysis diameters (medians of 17.3 and 18.0 mm, respectively), whereas strains from *spa* type t605 (*bap*+ high biofilm producer) showed the smallest hemolysis diameters

(median of 12.7 mm). Strain sa3154 was analyzed separately because it possesses 10 SAg genes (Table 1). Isolate sa3154 had much lower hemolytic activity than the rest of the t529 strains (Figure 2). A one-way ANOVA using Dunnett's multiple comparisons test showed statistical significance between t529 and t605 strains ($P = 0.024$) and this difference was confirmed with a more conservative Kruskal-Wallis test ($P = 0.043$).

Adherence to MAC-T bovine mammary epithelial cells was measured 30 min postinfection. As shown in Figure 3A, the ability of strains from all *spa* types to adhere to MAC-T cells was relatively similar. On the other hand, the level of intracellular persistence measured 20 h postinfection showed some differences among strains and *spa* types. Strains from *spa* type t2445 showed higher intracellular persistence compared with the strains of the predominant Canadian *spa* type t529 (one-way ANOVA and Tukey's multiple comparison test, $P = 0.03$; Figure 3B), but only a statistical trend was shown with the more conservative Kruskal-Wallis test ($P = 0.06$). It is interesting to note that strain sa3456 (t2445) showed the strongest ability to persist within MAC-T cells compared with all other strains although one value was particularly high (Figure 3B). High variability was observed among strains from several *spa* types including t267, t359, t2445 and t13401, indicating that the relative intracellular persistence ability of *Staph. aureus* strains may not be associated with their clonal origin. The exception was *spa* type t529 that clustered strains demonstrating a similar low-intracellular persistence pattern.

Colonization of Mouse Mammary Glands

Each mouse mammary gland was infected with *Staph. aureus* and glands were then collected 10 h postinfection to simulate an early stage infection and at 20 h to measure bacterial colonization in a well-established infection. No difference in the early infection levels was noted between the Canadian *spa* types included in this study (data not shown). However, as illustrated in Figure 4, significant differences were found in the levels of colonization at a later stage of infection. Because *Staph. aureus* strains are grouped into specific clonal lineages each possessing a remarkably identical genome because of a highly developed restriction-modification system that prevents, most of the time, horizontal gene transfer among lineages (Park et al., 2020), we considered that all isolates from the same *spa* type had an identical clonal origin and as such in Figure 4, each *spa* type is therefore represented by 10 to 12 repeats (2 strains per *spa* type, each colonizing 5–6 mammary glands). Excessive redness of the mammary glands was observed only with strains sa3511 and sa2539 (both *spa*

Figure 2. Diameters of *Staphylococcus aureus* β -hemolysis measured on blood agar plates for different *spa* types. Each dot corresponds to the mean value of the diameters of hemolysis recorded for each of the 23 *Staph. aureus* strains tested [3 replicates per strain, and the number of strains (n) per *spa* type is indicated]. The symbol colors are for the strains listed in the box and such strains are those that were used in all experiments including the mouse model of mastitis and evaluation of host cytokines (Figure 4–6). The clear symbols are for the other strains listed in Table 1. Strain sa3154 possessing 10 superantigen genes was analyzed separately. The horizontal bars correspond to the median for each *spa* type. The statistical test used for the analysis was a one-way ANOVA using Dunnett's multiple comparisons test. Each *spa* type was compared with *spa* type t529 acting as the reference * $P = 0.024$.

type t529). Those strains, similarly to strains from *spa* type t359, exhibited the highest bacterial loads recovered from mammary glands ($P < 0.006$). On the other hand, strain sa3154 (*spa* type t529), which possesses at least 10 SAg genes acquired by horizontal gene transfer (Table 1), showed a lower bacterial load than that seen with the 2 other strains of t529 ($P < 0.0001$). It should be noted that strains from the *spa* types that produced the highest amount of biofilm; that is, *spa* types t605 (*bap*+) and t13401 (*bap*–), had the lowest bacterial loads recovered from mammary glands (Figure 4).

Figure 3. Adherence and intracellular persistence of different *Staphylococcus aureus* *spa* types on and in bovine mammary gland epithelial cells (MAC-T). (A) Number of cfu adhered on MAC-T cell surface 30 min postinfection and corrected for viable cells (MTT optical density units, see Materials and Methods). (B) Number of colony-forming units recovered within MAC-T cells 20 h postinfection (also corrected for viable cells). Each dot corresponds to the mean count (cfu) recorded for each *Staph. aureus* strain [2 replicates per strain, and the number of strains (n) is indicated]. The symbol colors are for the strains listed in the box and such strains are those that were used in all experiments including the mouse model of mastitis and evaluation of host cytokines (Figure 4–6). The clear symbols are for the other strains listed in Table 1. The horizontal bars correspond to the median for each *spa* type. The statistical test used for the analysis was a one-way ANOVA using Tukey's multiple comparisons test. Each *spa* type was compared with all other *spa* types. * $P = 0.03$.

Inflammation of Mammary Glands During IMI

The MPO produced primarily by neutrophils, was monitored to measure inflammation of mammary glands during IMI caused by the different *spa* types of *Staph. aureus*. Figure 5 shows that IMI caused by strains from *spa* type t267 were correlated with a significantly higher amount of MPO than t529 ($P = 0.024$), suggesting a higher local inflammatory response in mammary glands likely due to better neutrophil recruitment. However, note that the 2 highest MPO values measured for strain sa3493 (t267) originated from 2 mammary glands (left and right) of the same mouse. Hence, measurement of MPO released by neutrophils could not detect any major difference between *spa* types.

Cytokines Analysis

Pro-inflammatory cytokines and chemokines are essential for the recruitment of immune cells at the site of infection and they modulate the inflammatory response

that normally leads to clearance of pathogens. Figure 6 shows the cytokines produced during IMI caused by each *Staph. aureus* *spa* type studied. The concentrations of 14 cytokines released in the mouse model of IMI were greater than 1,000 pg/g of glands (eotaxin, G-CSF, IL-1 α , IL-1 β , IL-6, IL-9, KC, LIF, LIX, MCP-1, MIP-1 α , MIP-1 β , MIP-2, VEGF). The concentrations of IL-1 β and IL-6 released during IMI with strains from *spa* types t605 and t13401 and by strain sa3154 (t529, 10 SAgS) were lower than those from other strains from *spa* types t529 and t2445. The concentrations of IL-1 α , LIX, MIP-1 α and MIP-1 β released by *spa* type t529 IMIs were lower than those induced by other *spa* types.

As we studied the expression of a large panel of inflammatory mediators following infection with 13 strains, PCA was used to determine the correlation between the bacterial *spa* types and the cytokine responses they induced (specific cytokines and concentrations). For each bacterial strain, cytokine profiles were obtained from 5 to 6 infected glands (10–12 glands per *spa* type). As shown in Figure 7, several cytokines can be

positively or negatively correlated with some *spa* types. Using PCA analysis, strains eliciting similar cytokine expression profiles (specific cytokines and their level of expression or concentration) were grouped together. The quarters shown in Figure 7 were used to aid in the visualization of positive, negative, and opposing correlations. Strains that elicited similar immune responses were clustered together into the same quarter, whether they belonged to the same *spa* type or not. In Figure 7, the 4 quarters (groups 1–4) are represented: both high biofilm producers (t605 and t13401 strains) and t267 strains were clustered in group 1, strains from the

Figure 4. Bacterial load in mouse mammary glands 20 h after IMI. Each dot corresponds to one infected mammary gland (5–6 glands per strain for a total of 10–12 glands infected per *spa* type). Each color represents all mammary glands infected by the same strain within each *spa* type (see strain identification in the box). Strain sa3154 possessing 10 superantigen genes was analyzed separately. Horizontal bars represent the median for each *spa* type. The statistical test used for the analysis was a one-way ANOVA using Tukey's multiple comparisons test. Each *spa* type was compared with all other *spa* types. No significant difference was observed between *spa* types with the same letter and *spa* types with different letters were significantly different ($P < 0.006$).

Figure 5. Inflammation levels as measured by myeloperoxidase (MPO) activity in infected mammary glands 20 h postinfection. The amount of MPO units released by neutrophils was used as a local inflammation marker. Each color represents all mammary glands infected by the same strain within each *spa* type (see strain identification in the box). Strain sa3154 possessing 10 superantigen genes was analyzed separately. Dulbecco's PBS (D-PBS) was injected for the mock infections (control mouse group without bacteria). Horizontal bars represent the median for each *spa* type. The statistical test used for the analysis was a one-way ANOVA using Tukey's multiple comparisons test. Each *spa* type was compared with all other *spa* types. No significant difference was observed between *spa* types with the same letter and *spa* types with different letters were significantly different ($P = 0.024$).

most prevalent *spa* type t529 (strain sa3154 excluded) were found in group 2, and strains from *spa* type t2445 were found in groups 2 and 3, whereas t359 strains were found in groups 1 and 4. In Figure 7, the vectors show the cytokine profiles that differentiate the host responses associated with the various strains. This means that strains that can harm the host or that produce a variety of virulence factors are expected to elicit strong

and specific cytokine responses. As a result, it is worth noting that the t529 strain sa3154, which possesses by far the greatest number of superantigens (10 SAgS), is found isolated in the center of the 4 quarters (i.e., no cytokine profile association), whereas all other strains could be associated with specific cytokine expression profiles. Group 1 showed a positive correlation with expression of G-CSF, KC, IL-5, LIF and eotaxin and a negative correlation for expression of IL-4, IL-12(p40) and IL-13 (Figure 7). For group 2, a positive correlation was observed for expression of IL-1 β , IL-6, M-CSF, and IL-12(p70), whereas a negative correlation was found with LIF (Figure 7).

DISCUSSION

Staphylococcus aureus is a pathogen with many geographical and host-specific clonal origins. Previous studies have identified 6 main *spa* types in Canadian dairy cattle: t529, t267, t359, t605, t2445, and t13401 (Said et al., 2010; Veh et al., 2015; Pichette-Jolette et al., 2019; Naushad et al., 2020). With the exception of t605, CC126, these *spa* types are from the CC151 and CC97, which are also the most widespread CC in Europe (Hoekstra et al., 2020). Some of our previous studies attempted to predict the duration of IMI based on the genotypic and phenotypic characteristics of *Staph.*

Figure 6. Cytokine concentrations detected in infected mammary glands 20 h postinfection. Each box corresponds to a range of concentrations (pg/g of mammary gland) measured for all mammary glands infected by all the strains within each *spa* type (2 strains for each *spa* types, additionally to strain sa3154, which possessed 10 superantigen genes and which was analyzed separately). This represented 10 to 12 glands per *spa* type. Results for strain sa3154 (t529) are from 6 glands. The darkest boxes represent the highest concentrations of cytokines measured in the mammary glands (see scale on the right). The crossed box corresponds to a high out-of-range value. Dulbecco's PBS (D-PBS) was injected for the mock infections (control mice group without bacteria; data from 4 glands).

aureus isolates from different quarter milk samples collected over 2 years from 91 farms (Veh et al., 2015; Pichette-Jollette et al., 2019). Such retrospective studies have only been partially successful in associating the phenotype or genotype of *Staph. aureus* isolates with the observed disease outcome in cows (clinical, subclinical, persistent). The reasons for this are multiple (herd, farm management, treatment, and level of host immune resistance and response). Similarly, a recent pan-genomic study on bovine *Staph. aureus* isolates from milk could not find any correlation between the virulence potential of isolates (total virulence genes minus core genome virulence genes) and SCC in milk as the proxy for disease severity (Naushad et al., 2020). Likewise, Hoekstra et al. (2020), could identify a variety of genes associated with clinical mastitis but many of these genes were sometimes also present in CC associated with a subclinical disease. Here, we have taken a step back to better characterize the phenotype and genotype of *Staph. aureus spa* types encountered in Canadian dairy

herds. We used in vitro and in vivo mastitis models that removed the “cow, farm, and management effects” from the analysis and demonstrated significant differences in the virulence potential of *Staph. aureus spa* types.

The ability of *Staph. aureus* to adhere, invade and persist in host cells allows the bacteria to escape the immune system and antibiotic therapies (Proctor et al., 2006). Both adherence and intracellular persistence abilities of the *Staph. aureus spa* types were investigated using the bovine mammary gland epithelial MAC-T cell line. No significant differences were observed for the adherence ability of the strains on the MAC-T cell surface, but some *spa* types showed differences in intracellular persistence. Only *spa* type t2445 resulted in significantly higher counts in MAC-T cells compared with those from *spa* type t529, which was inversely the least persistent. This property suggests that strains from t2445 could establish chronic IMI (Kerro Dego et al., 2002; Rainard et al., 2018). The high variability of persistence among *spa* types t267, t359, and t13401

Figure 7. Principal component analysis (PCA) of cytokine levels detected in infected mammary glands 20 h postinfection. Vectors representing the concentration of various cytokines measured in homogenates of infected mammary glands are shown in red. The length of each vector is associated with the cytokine concentration measured, and the angles indicate the positive or negative correlations between each cytokine. The biplot is combining *spa* types and vectors based on the PCA correlation. Strains from the same *spa* type are indicated by the same color scheme. Axes F1 and F2 are the principal components displaying the highest and second-highest percentage of variables (32.52% and 21.01%, respectively).

suggest that the clonal origin is not the only variable that needs to be taken into account for *Staph. aureus* persistence in epithelial cells. The hemolytic activity of each strain is an example of such variables because *Staph. aureus* α -hemolysin contributes to host cell lysis. Although MAC-T cell killing was considered in our intracellular persistence assay, strains from t529 were among the most hemolytic on blood agar plates (Figure 2) and were accordingly the least persistent inside cells (Figure 3B). Indeed, production of α -hemolysin leads to cell death and release of intracellular bacteria (Soe et al., 2021).

Our mouse model of IMI was used to investigate the ability of *Staph. aureus* strains to infect and colonize the mammary glands, to evaluate the local inflammation and to measure some markers of the host immune response (cytokines) induced by each *spa* type. The lactating mouse model is a well-established model for the investigation of host-pathogen interactions during IMI (Brouillette and Malouin, 2005). In our study, extensive tissue redness was observed only on mammary glands infected by strains sa3511 and sa2539 (both from *spa* type t529). Strains from *spa* type t529 carried more SAg genes than any of the other *spa* types (Table 1). Wilson et al. (2018) have shown that SAg expressed by *Staph. aureus* strains can stimulate different subfamilies of T lymphocytes with the consequence of overstimulating the immune system and could favor the occurrence of clinical cases of mastitis. Strains from *spa* type (t529), which is part of the sequence type ST151, carry the *egc* cluster of SAg (*seg*, *sei*, *sen*, *sem*, *seo*) (Wilson et al., 2018; Naushad et al., 2020; Park et al., 2020). This observation therefore appears to fit well with the high virulence of our t529 strains in the mouse mastitis model. On the other hand, the t529 strain sa3154 is part of ST351 and possesses the *Staph. aureus* pathogenicity island SaPI_{bov} carrying the SAg genes *tst_{bov}*, *sec_{bov}*, *seil_{bov}* in addition to the *egc* cluster for a total of at least 10 SAg genes (Table 1). Strain sa3154 is similar to the well-studied bovine strain RF122, and Wilson et al. (2018) showed that the 3 SAg encoded by SaPI_{bov} alone could activate 13 of 18 boV β T cell subfamilies in comparison to the activation of only 11 of 18 subfamilies by all the SAg of the *egc* cluster. This may indicate that strain sa3154 is better equipped for immune evasion but the reason why it showed less colonization and inflammation, and no redness of the gland in contrast to the other strains of t529 in our mouse mastitis model is unclear. One possible reason is that sa3154 showed the lowest hemolytic activity among *spa* type t529 strains (Figure 2). Hemolysins are associated with the presence of injuries on the epithelium of the mammary gland and local inflammation. Such tissue

injuries are known to promote the loss of tissue tightness which leads to the presence of blood in milk and contributes to the manifestation of the clinical signs of mastitis (Rainard et al., 2018).

The biofilm hyperproducer strains from *spa* types t605 and t13401 were the least able to multiply in mouse mammary glands (Figure 4). Those isolates do not carry the *egc* cluster or *lukM* coding for the bovine specific leukocidin LukMF', but they are still among the predominant *spa* types found in Canadian herds (Pichette-Jolette et al., 2019). These results suggest an inverse correlation between the amount of biofilm produced and the number of colony-forming units recovered from infected mammary glands. Similarly, Pereyra et al. (2017) found that a *Staph. aureus* isolate that produced a large amount of biofilm was associated with a lower likelihood of mammary gland injury. However, the type of immune response it triggered was consistent with chronic mastitis. These results suggest that strains from *spa* types t605 and t13401 are more likely to induce chronic mastitis compared with those from *spa* types t529 and t359, which are inversely the most virulent in our mouse model of IMI (Figure 4). Indeed, Pichette-Jolette et al. (2019) has previously shown that *spa* type t13401 was more likely to persist in cows compared with those from *spa* type t529 and t359.

Serine-aspartate rich (Sdr) proteins are important virulence factors in *Staph. aureus* (Foster et al., 2014; Foster, 2019). Because SdrC, SdrD and SdrE may play a variety of roles in pathogenesis (biofilm formation, adhesion to host molecules and immune evasion mechanisms) we attempted to correlate their presence or sequence divergence (Table 2 and Supplemental Figure S1) to the phenotypes we observed in this study. Among the principal observations, 3 *spa* types of the CC97 clade (t267, t2445, t359; all ST352) had all 3 genes (*sdrC*, *sdrD*, *sdrE*) in their genomes but the strains from these *spa* groups were not the most virulent in the mastitis mouse model (Figure 4). However, the *spa* type t13401 (also CC97 but ST2187) did not possess *sdrD* and *sdrE* and was the least virulent (Figure 4). Noteworthy, there was a deletion of 330 bases in the *sdrC* gene of t2445 strains occurring in a repeated region of the protein rather than in the functional region. Interestingly, this *spa* type (t2445) was the weakest biofilm producer among the *spa* groups analyzed (Table 1). Whether or not this deletion affects the formation of biofilm is difficult to tell at this time. All strains from t605 (CC126) and t529 (CC151) presumably had a nonfunctional SdrC protein since they show mutations leading to an early stop codon in *sdrC* (Table 2 and Supplemental Figure S1). Similarly, Xue et al. (2011) reported deletions and stop codons within the

sdrC genes of many *Staph. aureus* isolates. This means that *spa* types t605 and t529 only possess SdrE as member of the serine-aspartate rich proteins. Furthermore, 2 of the 7 strains of t529 had no SdrE, whereas the other 5 strains possessed an allelic variant of *sdrE*. This is intriguing because t529 was the most virulent *spa* type (Figure 4). This SdrE variant is characterized by a modified functional domain of the protein and was previously associated with strains leading to bone infections in humans (Tung et al., 2000; Tristan et al., 2003). However, the presence of this variant does not appear to enhance virulence in the mouse model. In fact, no significant difference was found between strain sa2539 (detaining the allelic variant) and strain 3511 having no SdrE (Figure 4).

Neutrophils are one of the first host immune cells to be recruited locally during an IMI. As a part of the innate immunity, they are involved in the phagocytosis of both infected cells and pathogens. They also lead to local inflammation by the release of MPO to kill extracellular bacteria, and by the release of several cytokines and chemokines involved in chemotaxis and regulation of inflammation. As shown in Figure 5, only one significant difference was observed with a higher amount of MPO released for strains from t267 compared with those from t529. Overall, however, measurement of MPO released by neutrophils could not detect any major difference between *spa* types.

Cytokines are involved in regulation of inflammation, immune cells recruitment and their activation. They are an important part of the host's ability to clear pathogens. However, in the case of chronic inflammatory diseases such as it is often the case with *Staph. aureus* IMI, a loss of pro- and anti-inflammatory cytokines regulation and a failure to induce a strong immune response could explain the occurrence of recurrent infections or the increase in duration of the disease. Our data show that all *Staph. aureus* strains can induce TNF- α , IL-1 β , and IL-6, which are involved in innate immune responses (Figure 6). A consistent level of IFN- γ observed for all strains also suggests that the NK cells were activated similarly to other immune cells involved in the innate response (Figure 6). In addition, the high MCP concentration measured in glands (Figure 6), suggests that all strains can recruit inflammatory macrophages from the bone marrow. The statistical analysis by PCA allowed some strains to be clustered based on the cytokine expression profiles they induced. High biofilm producers (t605 and t13401) and strains from t267 (i.e., group 1, Figure 7), led to profiles that were correlated with several cytokines involved in immune cells recruitment (eotaxin, KC), neutrophil proliferation (G-CSF), B lymphocyte proliferation (IL-5), inflammation (KC, LIF), and healing (KC) (Figure

7). Strains sa3511 and sa2539 of *spa* type t529 (CC151, ST151), such as group 2 (Figure 7), induced cytokines involved in pro-inflammatory response and fever (IL-1 β , IL-6), monocytes proliferation (GM-CSF) and the naïve T lymphocyte differentiation in Th1 (IL-12; Figure 7). As reported by Oviedo-Boyso et al. (2007), the immune response induced during *Staph. aureus* IMI (leading to clinical or subclinical mastitis) is associated with an increase in milk SCC, an increase of IL-1 β , IL-12, TNF- α , C5a, and IgG2 concentrations, and the recruitment of CD8⁺ lymphocytes. Thus, this immune response is similar to that we have observed for group 2 strains from t529 with the exception of strain sa3154 (CC151, ST351) possessing more SAg genes, and which was not associated with any specific cytokine expression profile. IL-1 β is produced by macrophages and epithelial cells and has previously been associated with the first stage of *Staph. aureus* IMIs (Oviedo-Boyso et al., 2007). Interleukin-6, also produced by macrophages, has been associated with septic shock during the acute phase of *Staph. aureus* infections and facilitate the replacement of neutrophils to prevent deleterious effects of inflammation (Oviedo-Boyso et al., 2007). In addition, IL-12 can stimulate the production of IFN- γ and TNF- α (both pro-inflammatory cytokines) from Th1 lymphocytes and NK cells. Interleukin-12 may also enhance cytotoxicity by both NK cells and Tc CD8⁺ lymphocytes, act as a mediator between acquired and innate immunity, and may be involved in the regulation of lymphocyte differentiation. These observations suggest that most strains from t529 may lead to acute mastitis with severe symptoms. Similarly, cytokines IL-1 β , IL-12 and TNF- α were associated with clinical mastitis caused by several other bacterial pathogens such as *Streptococcus uberis*, *Escherichia coli*, *Klebsiella pneumoniae*, and *Pseudomonas aeruginosa* (Oviedo-Boyso et al., 2007).

This study examined proxies for host-pathogen interactions led by the major *Staph. aureus spa* types responsible for IMI in Canadian dairy herds using in vitro and in vivo mastitis models. These models enabled the search for associations between *spa* types and their ability to invade and persist in both mammary epithelial cells and mouse mammary glands, though our interpretations and possible extrapolation of results to cows should be used with caution. Also, the host (mouse) immune response induced by *Staph. aureus* strains of different clonal origins was measured and analyzed. Results show that most strains from the *spa* type t529 (CC151, ST151) have characteristics that are often correlated with clinical mastitis cases, whereas characteristics of strains from *spa* types t267, t605, and t13401 are generally correlated with subclinical or chronic mastitis. The exception was with strain

sa3154, which is part of the t529 *spa* type but of the sequence type ST351. This strain possesses at least 10 SAg genes and produced a moderate level of infection similar to t267 and induces a low immune response. Isolates positive for *tst* such as sa3154 are however rare among t529 isolates in Canada (9/115 isolates [8%]; Pichette-Jolette et al., 2019). Based on the phenotypic data collected in this study, the virulence potential of *Staph. aureus* strains can be ranked as follows:

$$\text{t529 (ST151)} > \text{t359} > \text{t2445} > \text{t529 (ST351)} = \\ \text{t267} = \text{t605} > \text{t13401}.$$

This is in agreement, in part, with our retrospective study of bovine mastitis cases which showed that strains from t529 and t359 were less likely to cause persistent IMI compared with strains from t13401 and that clinical mastitis was more often associated with *lukM* positive strains (Pichette-Jolette et al., 2019). Strain sa3154, which carries both the *egc* cluster of SAgS as well as SaPIbov (*tst_{bov}*, *sec_{bov}*, *seil_{bov}*) and appears to be less virulent than other t529 strains or at least in the mouse model, is an exception. Retrospectively, it would have been nice to include more *tst*-positive strains in our work to corroborate findings on strain sa3154 but at the onset of this study, we did not realize how different this strain would be from the other t529 strains. This type of strain could be easily identified by a PCR of the *tst* gene.

Thus, identification of the *spa* type at an early stage in the diagnosis of *Staph. aureus* IMI might help to support management decisions based on a certain prediction of disease outcome. The observations and assumptions made here should now be validated in studies where the *Staph. aureus* lineages of interest are randomly assigned to a standardized cow population to assess and verify clinical outcomes. Although *spa* typing is simple, an equivalent rapid and cost-effective alternative method for typing strains has also been described (ribosomal-spacer PCR; Fournier et al., 2008; Boss et al., 2016). Predicting the outcome of the disease by determining the clonal lineage of isolates could significantly reduce the economic losses caused by IMI in bovine dairy herds.

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